Exhibit B

CLINICAL AND DIAGNOSTIC LABORATORY IMMUNOLOGY, July 2003, p. 596–601 Vol. 10, No. 4
1071-412X/03/$08.00+0 DOI: 10.1128/CDLI.10.4.596–601.2003

High-Level Expression of Truncated Surface Antigen P50 of *Babesia gibsoni* in Insect Cells by Baculovirus and Evaluation of Its Immunogenicity and Antigenicity

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Received 9 January 2003/Returned for modification 25 February 2003/Accepted 20 March 2003

Previously, we identified an immunodominant antigen, P50 of *Babesia gibsoni*. In the present study, the gene encoding the truncated P50 (rP50t) without a C-terminal hydrophobic region (29 amino acids [aa]) was expressed in insect cells by a recombinant baculovirus. The highly hydrophobic C-terminal 20-aa regions seems to be a transmembrane region, which was evidenced by the fact that rP50t was effectively secreted into the supernatant of insect cells infected with the recombinant baculovirus. N-terminal amino acid sequence analysis of rP50t indicated that N-terminal 19 aa function as a signal peptide. The expression level of rP50t reached up to 2 mg per 10^8 cells infected with the recombinant baculovirus. The immunogenic property of rP50t was evaluated by an immunization test in mice. Mice immunized with rP50t induced a high-level antibody titer against the *B. gibsoni* merozoite. Monoclonal antibodies (MAbs) to rP50t were produced in mice to determine the immunogenic regions of P50. The epitope(s) recognized by all five MAbs were located between aa 190 and 273, suggesting that the central part of P50 is a highly immunogenic region. The diagnostic potential of rP50t was evaluated using an enzyme-linked immunosorbent assay (ELISA). The ELISA was able to differentiate clearly ($P < 0.0001$) between *B. gibsoni*-infected dog serum and *B. canis*-infected dog serum or noninfected dog serum. Our results indicated that the rP50t may provide a useful potential immunogenic reagent for use in diagnosis and as a subunit vaccine to control *B. gibsoni* infection in dogs.

Babesia gibsoni is a tick-borne hemoprotozoan parasite that causes piroplasmosis in dogs. The disease is characterized by remittent fever, progressive anemia, hemoglobinuria, and marked splenomegaly and hepatomegaly; sometimes, it causes death (2, 22, 26). *B. gibsoni* infection is endemic in many regions of Asia, Africa, Europe, and America (11, 14). Recently, this disease has frequently been observed in companion animals, becoming a significant problem from a clinical point of view (1, 4, 16, 20).

The development of a vaccine that would reduce or prevent the clinical symptoms of canine *B. gibsoni* infection is considered to be the best approach for controlling the disease. However, no vaccine is currently available. Therefore, there is a need to develop an effective vaccine to control *B. gibsoni* infection in dogs. Further, the development of a diagnostic method for canine *B. gibsoni* infection was also considered important for controlling this infectious disease. Previously, we cloned a novel gene encoding a protein with a molecular mass of 50 kDa (P50) from *B. gibsoni* and demonstrated that P50 is a major surface protein and an immunodominant antigen (6). The entire recombinant P50 (rP50) expressed in insect cells by the baculovirus was shown to have good antigenicity and immunogenicity (6). The enzyme-linked immunosorbent assay (ELISA) with rP50 as an antigen could detect the specific antibody in the sera from dogs experimentally infected with *B. gibsoni*. However, its usefulness was hindered by a large contamination with many proteins from insect cells or baculovirus.

It is known that a truncated form of a membrane protein without a transmembrane region can be secreted into a supernatant of insect cells infected with recombinant baculoviruses and, therefore, provide an easy way to prepare pure immunogenic proteins (19). In the present study, the gene encoding truncated P50 (rP50t) without a C-terminal hydrophobic transmembrane region was expressed in insect cells by the recombinant baculovirus in order to obtain a pure and large amount of rP50t from the culture supernatant. Furthermore, we evaluated the possibility that the rP50t could be used as an immunogen for the animals and as an antigen for the ELISA to diagnose canine *B. gibsoni* infection.

MATERIALS AND METHODS

Parasite. The NRCPD strain (6, 12) of *B. gibsoni* parasite was used. *B. gibsoni*-infected red blood cells (RBC) were collected from an experimentally infected dog at peak parasitemia (25% parasitemia) and washed twice with phosphate-buffered saline (PBS) by centrifugation at 1,200 × *g* (himac CF7D2; Hitachi, Tokyo, Japan) for 10 min at 4°C: then, the infected RBC were kept at −80°C until use. Fresh *B. gibsoni* parasites were prepared from canine RBC-substituted SCID (Ca-RBC-SCID) mice (Clea Japan, Tokyo, Japan) infected with *B. gibsoni* (5).

Computer analysis of the structure of the P50. Computer analysis of the signal sequence and the transmembrane region of the P50 was performed with the computer program SOSUI (http://sosui.proteome.bio.tuat.ac.jp/sosuiframe0) (8, 17). The antigenicity or hydrophilicity of the P50 was analyzed by the Welling

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method (21) or the Hopp-Woods method (9, 10) with the computer program Mac Vector 6.5.3 (Oxford Molecular, Hunt Valley, Calif.).

Construction of the two recombinant baculoviruses AcP50 and AcP50t. Construction of the recombinant baculovirus AcP50 carrying a complete open reading frame of the *B. gibsoni* P50 gene was described in a previous paper (6). One set of oligonucleotide primers including the *Eco*RI restriction enzyme site for PCR amplifying the truncated gene encoding P50 without the C-terminal 29 amino acids (aa) was designed (forward, 5'-ACGAATTCTAATATGAATG TCGTT-3'; reverse, 5'-ACGAATTCTGGAGCTTCTGCACGT-3'). The PCR product was digested with *Eco*RI and then ligated into the *Eco*RI site of the baculovirus transfer vector pBacPAK8 (Clontech, Palo Alto, Calif.). The structure of recombinant plasmid pBP50t was checked by restriction enzyme analysis, and the nucleotide sequence was analyzed with a model ABI 3100 DNA sequencer (Applied Biosystems, Foster City, Calif.). Construction of the recombinant baculovirus AcP50t was performed as described previously (23–25).

Expression of the rP50 and rP50t in insect cells. High Five (HF) cells infected with AcP50 or AcP50t (10 PFU/cell) were incubated with 1 ml of a protein-free Sf-900 II SFM medium (Gibco BRL, Rockville, Md.) for 4 days. After incubation, the cell and culture medium mixtures were centrifuged at 1,400 × *g* (himac CF15D2; Hitachi) for 5 min at 4°C, and the supernatants were further centrifuged at 99,000 × *g* (himac CS150GE; Hitachi) for 2 h at 4°C to get rid of the viruses. The resulting supernatants were collected and used for further experiments. The infected cells were washed twice with PBS by centrifugation at 5,000 rpm for 5 min at 4°C and then resuspended in 1 ml of PBS for further analysis.

SDS-PAGE and Western blotting. The HF cells infected with AcP50 or AcP50t and its supernatants were mixed with an equal volume of a 2× sodium dodecyl sulfate (SDS) gel-loading buffer (100 mM Tris-HCl [pH 6.8], 100 mM 2-mercaptoehanol, 4% SDS, 0.2% bromophenol blue, 20% glycerol). The purification of *B. gibsoni* merozoites was performed as previously described (15). The purified *B. gibsoni* merozoites were mixed with an equal volume of an SDS gel-loading buffer under reducing conditions. The samples were boiled for 5 min, and then each 10 μl of sample was subjected to SDS-polyacrylamide gel electrophoresis (PAGE). For SDS-PAGE analysis, the gel was stained with Coomassie brilliant blue (CBB). After SDS-PAGE, the protein bands in the gel were electrically transferred to a membrane (Immobilon transfer membrane; Millipore, Bedford, Mass.). Western blotting was carried out as described previously (6, 24). The signals were detected with enhanced chemiluminescence detection reagents (Amersham Biosciences, Little Chalfont, United Kingdom) using the VersaDoc 5000 imager (Bio-Rad, Hercules, Calif.).

N-terminal amino acid sequence analysis of rP50t. The membrane transferred with the rP50t described above was stained with CBB, and the protein bands were excised and subjected to N-terminal amino acid sequence analysis. The amino acid sequence analysis was performed with the HP G1005A protein sequencing system (Hewlett-Packard, Palo Alto, Calif.) following the manufacturer's instructions.

IFAT. Indirect immunofluorescence antibody test (IFAT) was carried out as described in our previous work (6).

Determination of antibody titer by ELISA with GST-P50. The entire P50 gene in the pBluescript SK(+) vectors was subcloned into the pGEX-4T-3 plasmid (Promega, Madison, Wis.) of the bacterial expression vector and designated as pGEX/P50. The recombinant protein was expressed as the fusion protein of the glutathione *S*-transferase (GST) protein in the *Escherichia coli* DH5α strain according to the manufacturer's instructions (Promega) and designated GST-P50. The purification of GST-P50 was performed as described previously (13). The ELISA with GST-P50 was carried out according to a method described earlier (6, 25).

Production of monoclonal antibodies (MAbs) against P50. The supernatant containing rP50t was concentrated 10 times using a Vivapore 10/20 7,500-molecular-weight-cutoff polyethersulfone membrane (Vivascience, Stonehouse, United Kingdom). One hundred micrograms of the rP50t in Freund's complete adjuvant (Difco Laboratories, Detroit, Mich.) was intraperitoneally injected into mice (BALB/c, 8 weeks old). The same antigen in Freund's incomplete adjuvant (Difco) was intraperitoneally injected into the mice on day 14 and again on day 28. The mouse with the highest antibody titer by the ELISA with GST-P50 was selected as the spleen donor. Splenocytes (1.25×10^8 cells) were fused with Sp2 myeloma cells (10^7), and hybridomas were cultured in a GIT medium (Wako, Tokyo, Japan) supplemented with hypoxanthine, aminopterin, and thymidine in 96-well plates. Two to three weeks after fusion, screening of hybridomas was done by the ELISA with GST-P50. Positive hybridomas were cloned by limiting dilution.

Characterization of MAbs against P50. To determine the epitope(s) recognized by MAbs, two kinds of deletion clones of P50 were constructed and expressed in *E. coli* as the GST fusion protein (Fig. 1C). The GST-P50 encodes the entire open reading frame of the P50 gene described above (aa 1 to 466). The GST-P50B encodes a peptide from the start codon to the *Bgl*II restriction enzyme site (aa 1 to 273) of the P50 gene. The GST-P50N encodes a peptide from the start codon to the *Nde*I restriction enzyme site (aa 1 to 190) of the P50 gene. The three fusion proteins and the control GST were used for the antigen in dot blotting. Each 1 μg of recombinant proteins was spotted onto the nitrocellulose membrane and then blocked with 3% skim milk–PBS. The antibody reaction and signal detection were performed as in the method for Western blotting. The isotype of the MAbs was determined with a mouse MAb isotyping kit (Amersham) following the manufacturer's instructions.

Diagnosis of the canine *B. gibsoni* infection by ELISA with rP50t. Individual wells of a microtiter plate were coated with the rP50t (50 ng/well) in an antigen-coating buffer (0.05 M carbonate-bicarbonate buffer, pH 9.6). The ELISA was performed as described previously (6, 25). The canine sera used for ELISA were as follows: (i) sequential serum samples from a dog experimentally infected with *B. gibsoni* (NRCPD strain); (ii) 19 serum samples from dogs experimentally infected with *B. gibsoni*; (iii) 5 serum samples from dogs experimentally infected with *B. canis*; (iv) 17 serum samples from field dogs from clinical hospitals in Japan that had tested positive for *B. gibsoni* by microscopic examination or PCR analysis (7); (v) 24 serum samples from healthy dogs. All serum samples were used in a 200-fold dilution for ELISA. The cutoff value of 0.0463 was calculated from the results of the ELISA of 24 healthy-dog serum samples as follows: 0.0203125 (mean value) + 3 × 0.0086625 (standard deviation). The difference of the ELISA values between the group of sera from healthy dogs and other groups were compared statistically by nonparametric analysis using the Mann-Whitney U test.

Antiserum and antibodies. Production of antiserum against the gene 10-P50 was described in a previous work (6). Horseradish peroxidase-conjugated goat anti-mouse immunoglobulin G1 (IgG1) or IgG2a antibody (Bethyl Laboratories, Montgomery, Tex.) was used for the ELISA with the GST-P50 and Western blotting. Horseradish peroxidase-conjugated goat anti-dog IgG antibody (Bethyl) was used for the ELISA with the rP50t. Alexa-conjugated goat anti-mouse IgG antibody (Molecular Probe, Eugene, Oreg.) was used for IFAT.

All experiments described in this article were conducted in accordance with the Guiding Principles for the Care and Use of Research Animals promulgated by Obihiro University of Agriculture and Veterinary Medicine.

RESULTS

Construction of the recombinant baculovirus expressing rP50t. To design a truncated clone of the P50 gene, the amino acid sequence of P50 was analyzed by computer programs (9, 10). The result indicated that the P50 possessed a putative signal peptide (19 aa) and a transmembrane region (20 aa) on the N and C termini, respectively (Fig. 1A). Hence, the recombinant baculovirus AcP50t expressing the truncated P50 (rP50t) without the putative C terminus transmembrane region was constructed.

Expression and characterization of rP50t in insect cells. The HF cells or its culture medium infected with AcP50t expressing rP50t or AcP50 expressing rP50 was analyzed by SDS-PAGE and Western blotting. In the SDS-PAGE analysis, only the single band of the r50t with a molecular mass of 46 kDa was detected (Fig. 2A). In the Western blotting, the r50t with a molecular mass of 46 kDa was detected in both the culture medium and the infected cells (Fig. 2B). In contrast with rP50t, the entire rP50 with a molecular mass of 50 kDa was only detected in the infected cells but not in the culture medium (Fig. 2B). This result indicated that the C-terminal hydrophobic region (20 aa) of P50 functions as a transmembrane region anchoring to the cell membrane (Fig. 3). On the other hand, the N-terminal amino acid sequence of rP50t was found to correspond to aa 20 to 26 deduced from the P50 gene sequence (Fig. 3). This result indicated that the first 19 aa of P50 functions as a signal sequence. The expression level of rP50t in the culture medium reached 2 mg per 10^8 cells (data not shown).

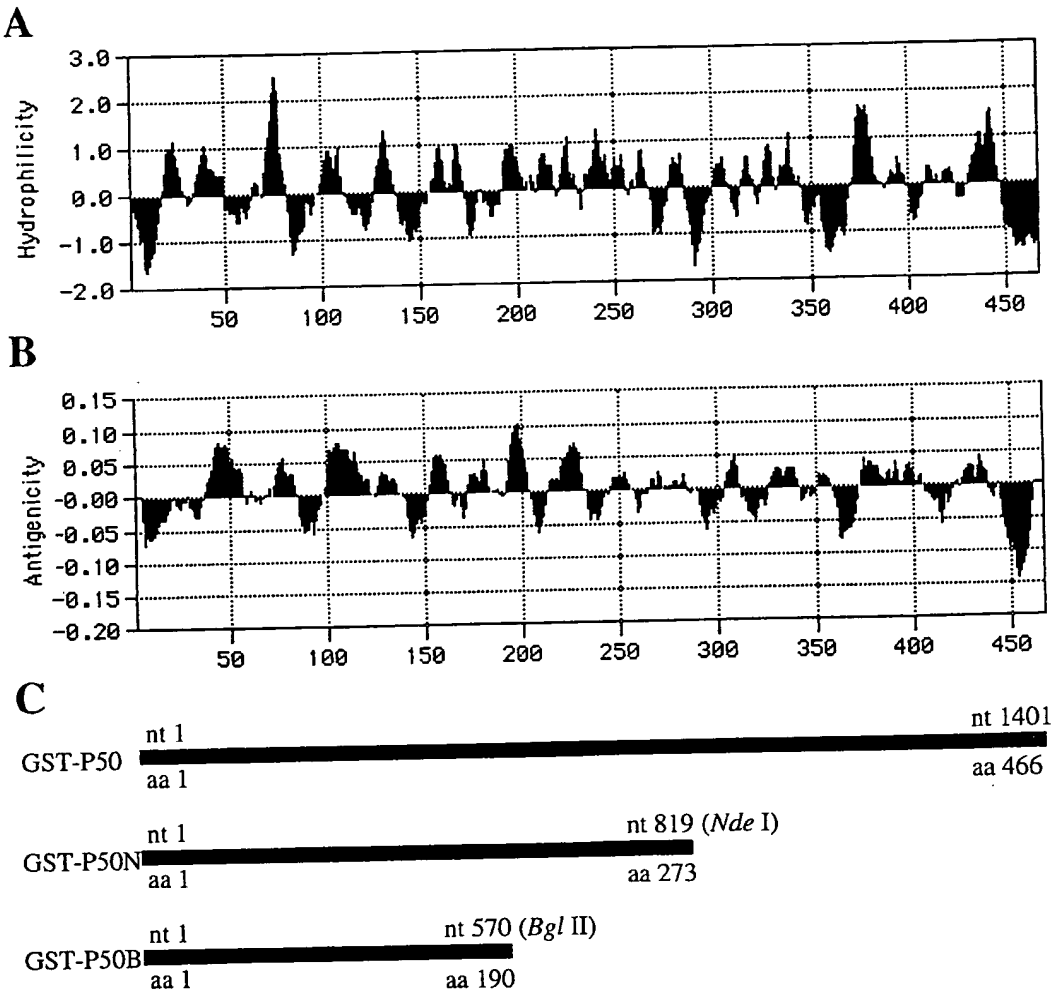

FIG. 1. Computer analysis of P50 or its nucleotide sequence. (A) Hydrophilicity analysis of the P50 amino acid sequence using the Hopp-Woods method. (B) Antigenicity analysis of the P50 amino acid sequence using the Welling method. The numbers under panels A and B show the position of the amino acid sequence. (C) Schemata of GST-P50 and two kinds of P50 deletion mutants expressed in *E. coli*, GST-P50N, and GST-P50B, used for the determination of the epitope(s) of MAbs on P50.

Analysis of the immunogenic properties of rP50t. The immunogenic activity of rP50 expressed in insect cells by the recombinant baculovirus was examined by inoculating the purified rP50t into mice. All three mice immunized with rP50t produced high titers (1:409,600 to 1:819,200) of specific antibody to GST-P50 (data not shown). The antibody to rP50t induced in mice reacted strongly with intact *B. gibsoni* merozoites, as judged by IFAT (Fig. 4). The control serum from a mouse immunized with insect cells infected with the recombinant baculovirus expressing β-galactosidase did not show any reactivity to *B. gibsoni* merozoites (Fig. 4).

Production and characterization of MAbs against rP50t. Five clones of hybridoma-secreting antibody against P50 were obtained and designated 1A3, 31D, 1F, E6, and 2D5. All MAbs reacted strongly to the P50 of *B. gibsoni* merozoites in Western blotting (Fig. 5). On the other hand, all MAbs specifically reacted with intact *B. gibsoni* merozoites, as judged by IFAT (data not shown). The epitopes recognized with the MAbs were analyzed by dot blotting using deletion clones of P50 expressed in *E. coli*. All MAbs reacted to the GST-P50 (aa 1 to 466) and GST-P50B (aa 1 to 273) but not to the GST-P50N (aa 1 to 190) and control GST (data not shown). This result indicated that the epitope(s) recognized with all five MAbs are located between aa 190 and 273 of P50.

ELISA with rP50t for diagnosing canine *B. gibsoni* infection. To evaluate whether the rP50t can be a suitable antigen for the diagnosis of *B. gibsoni* infection in dogs, the rP50t was tested in ELISA. As shown in Fig. 6, all serum samples from dogs experimentally infected with *B. gibsoni* were positive (optical density > 0.0463; $P < 0.0001$), whereas the serum samples from healthy dogs (optical density < 0.0463) and *B. canis*-infected dogs (optical density < 0.0463; $P = 0.2851$) were negative. Furthermore, all serum samples collected from the field dogs infected with *B. gibsoni* were positive (optical density > 0.0463; $P < 0.0001$). A dog experimentally infected with *B. gibsoni* developed a significant level of antibody response to the P50 antigen by day 8, as determined by the ELISA (Fig. 7). The antibody response was maintained at high levels until 360

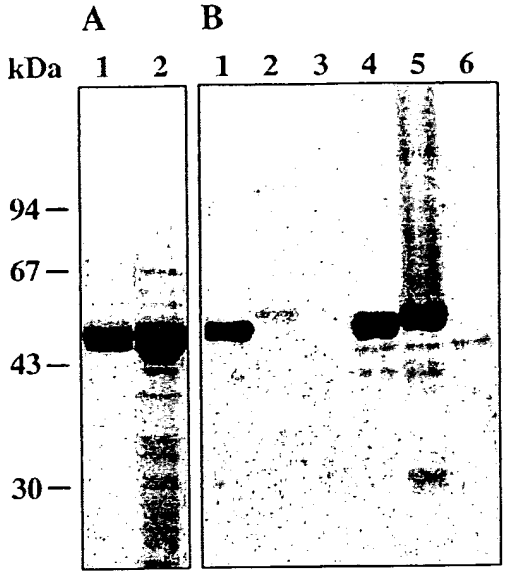

FIG. 2. SDS-PAGE and Western blot analysis of rP50t expressed in insect cells. (A) SDS-PAGE analysis of the rP50t expressed in insect cells. The protein bands were stained with CBB. Lane 1, culture medium of recombinant baculovirus AcP50t-infected cells expressing rP50t; lane 2, lysate of recombinant baculovirus AcP50t-infected cells expressing rP50t. (B) Western blot analysis of rP50t expressed in insect cells. Lanes 1 to 3, culture medium of recombinant baculovirus-infected cells; lanes 4 to 6, lysate of recombinant baculovirus-infected cells; lanes 1 and 4, AcP50t expressing rP50t; lanes 2 and 5, recombinant baculovirus AcP50 expressing rP50; lanes 3 and 6, control recombinant baculovirus AcLacZ expressing β-galactosidase.

days postinfection, even when it became the chronic stage of infection that characterized a significantly low level of parasitemia (Fig. 7).

DISCUSSION

In the previous study, we identified and cloned a novel gene encoding P50 from *B. gibsoni* by immunoscreening of a cDNA

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MNVVRSFLFFPIAFSLVRA▼NGEGKTAEATPAGTSTPTEPKA   40
AEAAPKAVDAAAVTFKQYLDFAMKLNEAVTLREEDTRKKLLV   80
NFPLFGAPPFDGAWGDLKDLLKKVTELRALLLKGHTFGLPAA  120
TTTDKQQQDANQTVGALFDFIVGVATDAVTVADKATRAVTGM  160
DPDKAVGFHVTPATADALFEFVPDLYEKLKDLHSKVGEWVEI  200
KSTFDDTKLVTQAGDHRPKHWLRQGGFTDQEVKGDTTLETLK  240
TKLGELVGPTKPCEKVLCTLASYALMKTPQDAAGKQAWIFLL  280
ASAMNNNAMKAKLEVAVNAVTPGKGETPVNQLKEVGKSLQLP  320
KEQVPKQYRFPGVYANLDVQHFWTVLTGVFGTILTDLEVDEK  360
DAQGKAGQVATRVAELVKVEGPLHSLTVQVAEMTKAGAGAGG  400
EAPAQAAAGTAGARAEAPAKEGQGEDGAHFCGIGMTVFFVSV  460
VIAVF                                        465
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FIG. 3. Amino acid sequence of P50. The N-terminal amino acid sequence in boldface type shows the signal peptide region (aa 1 to 19), and the underlined sequence shows the predicted sequence by the N-terminal amino acid sequence analysis of rP50t. The arrowhead shows the predicted carving site of the signal sequence. The C-terminal amino acid sequence in boldface type shows the truncated region of rP50t, and the underlined sequence shows the predicted transmembrane region as analyzed by SOSUI.

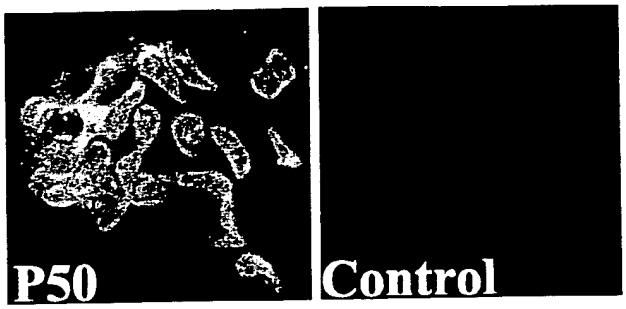

FIG. 4. IFAT analysis of the reactivity of serum collected from mice immunized with the rP50t against *B. gibsoni* merozoites. P50, anti-rP50t mouse serum; control, anti-β-galactosidase mouse serum.

library with infected dog serum and demonstrated that P50 is a surface membrane protein which showed no homology to the other proteins, including apicomplexan parasite, and P50 is considered to be an important candidate for the development of a diagnostic reagent or subunit vaccine to control *B. gibsoni* infection in dogs, although its function is unknown (6). In the present study, the gene encoding the truncated P50 without a putative C-terminal transmembrane region was expressed in insect cells by a baculovirus, and its immunogenicity and antigenicity were evaluated.

The baculovirus expression system offers significant advantages over prokaryotic and other eukaryotic systems for the production of proteins because, in many cases, the recombinant proteins expressed in insect cells by baculoviruses are present in high amounts and are similar in structure, biological activity, and immunological reactivity to the naturally occurring proteins. The advantages of the baculovirus expression system led us to express P50 in insect cells by a baculovirus. The rP50t without the putative C-terminal transmembrane region (29 aa) was effectively secreted into the insect cell culture medium, in contrast with the entire rP50, which was expressed as an intracellular protein. This result indicated that the C-

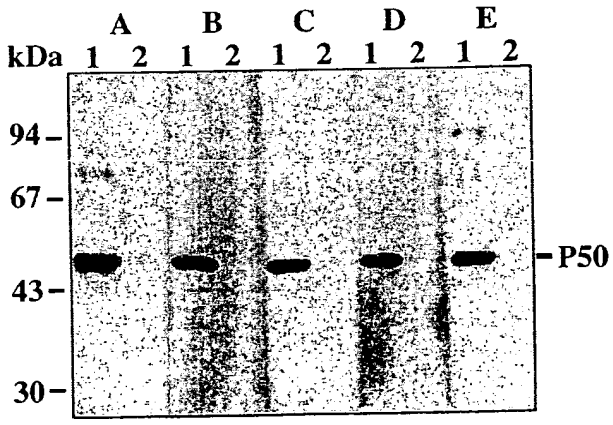

FIG. 5. Western blot analysis of reactivity of MAbs against P50. The reactivity of the MAbs to the native P50 protein was determined using *B. gibsoni* merozoite lysate as the antigen. Membrane A, MAb 1A3; membrane B, MAb 31D; membrane C, MAb E6; membrane D, MAb 1F; membrane E, MAb 2D5. Lanes 1, lysates of *B. gibsoni*-infected RBC; lanes 2, control lysates of healthy-dog RBC were used for the antigen.

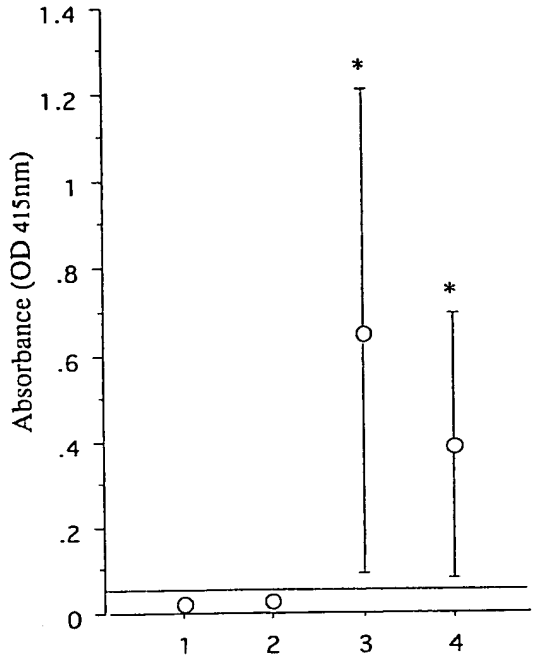

FIG. 6. Values from ELISA with rP50t and *B. gibsoni*-infected dog sera. The results are shown as mean values, and error bars represent standard deviations. Lane 1, sera from healthy dogs (n = 24); lane 2, *B. canis*-infected dog sera (n = 5); lane 3, experimentally *B. gibsoni*-infected dog sera (n = 19); lane 4, naturally *B. gibsoni*-infected dog sera (n = 17). A significant difference (*, $P < 0.0001$) between lane 1 and lane 3 or 4 was found.

terminal 20 aa functions as a transmembrane domain. On the other hand, the N-terminal amino acid sequence of rP50t corresponded to aa 20 to 26 deduced from the P50 gene sequence (6). This result indicated that the N-terminal 19 aa functions as a signal sequence.

The secreted rP50t offers some advantages over the intracellular rP50. The procedure for the preparation of secreted rP50t is much easier and overcomes the problem of contamination with proteins from insect cells or baculoviruses. In addition, the expression level of rP50t in the culture medium reached 100 mg/liter. This result indicated that the expression of the rP50t in insect cells was considered as a useful method for the preparation of the immunogen.

Mice were immunized with rP50t in order to determine the immunogenicity of the reagent. All mouse sera showed significantly high levels of antibody titer against GST-P50, and the sera were strongly reacted to the *B. gibsoni* merozoites in IFAT. These results suggested that the rP50t is a useful reagent as an immunogen for animals. Furthermore, MAbs to the rP50t were produced in mice to determine the immunogenic regions of P50. All five MAbs were confirmed to react strongly to intact blood merozoites of *B. gibsoni*. The epitope(s) recognized by all five MAbs was identified between aa 190 and 273, which suggested that the central part of P50 is a highly immunogenic region.

In our previous study, the entire intracellular rP50 expressed in insect cells was shown to have good antigenicity. Furthermore, the ELISA with rP50 was able to differentiate between *B. gibsoni*-infected dogs and normal dogs or *B. canis*-infected dogs, although the recombinant antigen was still contaminated with many proteins from insect cells or baculoviruses (6). Thus, the ELISA with rP50 showed that the high-level background might be caused by a nonspecific reaction to some contaminants. Therefore, we had to prepare an antigen control when detecting the antibody against P50 by the ELISA with rP50. In this study, the ELISA with the rP50t showed a significantly low level of background. This result suggested that the rP50t contained a significantly low level of the contaminated proteins from insect cells or baculoviruses, resulting in the low-level background. Therefore, the ELISA with the rP50t could specifically detect the antibody against P50 from the sera collected from dogs experimentally or naturally infected with *B. gibsoni* without the antigen control. In the sequential serum samples derived from a dog experimentally infected with *B. gibsoni*, ELISA antibody titers became positive at 8 days and increased thereafter until 360 days postinfection. This result was similar to the result of the ELISA titer with the rP50 as an antigen (6). These results indicated that the rP50t could be used as a more effective diagnostic antigen for the diagnosis of *B. gibsoni* infection in dogs.

Recently, baculoviruses have become a popular vehicle for the expression of genes from protozoan parasites in insect cells, and many reports have shown that animals immunized

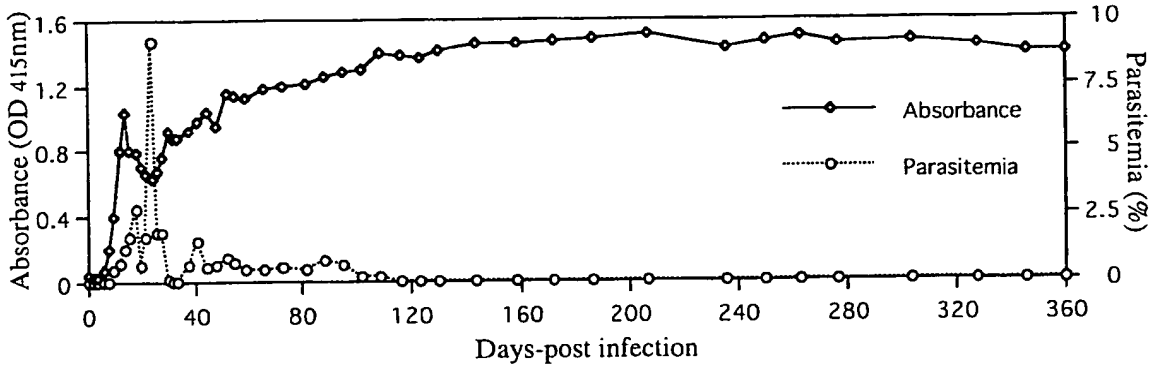

FIG. 7. Detection of antibody to rP50t in a dog experimentally infected with *B. gibsoni* by ELISA. The parasitemia is also shown in the same panel. The mean values from absorbance for two individual experiments are shown. OD, optical density.

with the recombinant antigens induced protective immunity against virulent parasite challenge infections (3, 18). In the present study, mice immunized with rP50t expressed in insect cells induced a high titer of antibody against the blood merozoite of *B. gibsoni*, although its protective effects had not been investigated. Eventually, we will study the feasibility of rP50t as a subunit vaccine to control *B. gibsoni* infection in dogs.

ACKNOWLEDGMENTS

This work was supported by a grant from The 21st Century COE Program (A-1) and a Grant-in-Aid for Scientific Research (13556049), both from the Ministry of Education, Science, Sports, and Culture of Japan. S.F. is supported by a Research Fellowship of the Japan Society for the Promotion of Science for Young Scientists.

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